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PROVISIONAL APPLICATION COVER SHEET

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INVENTOR(s)/APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
HERSCOVICS	Annette	A.	4837 Hutchison St., #5, Montréal, Québec, Canada H2V 4A4
TREMBLAY	Linda	O.	5156 Pierre Tétreault, Montréal, Québec, Canada H1K 2Y8

TITLE OF THE INVENTION *(280 characters max)*

α1,2-MANNOSIDASE AND THERAPEUTICAL USES THEREOF

CORRESPONDENCE ADDRESS

France Côté
SWABEY OGILVY RENAULT
1981 McGill College Avenue, Suite 1600, Montréal

STATE	Québec	ZIP CODE	H3A 2Y3	COUNTRY	Canada

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted,

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PROVISIONAL APPLICATION FILING ONLY

α1,2-MANNOSIDASE AND THERAPEUTICAL USES THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a novel α1,2-mannosidase and its therapeutical uses thereof in the treatment of genetic diseases.

(b) Description of Prior Art

α1,2-Mannosidases are essential for hybrid and complex *N*-glycan biosynthesis in mammalian cells (Herscovics, 1999). Following the removal of the glucose residues from the $Glc_3Man_9GlcNAc_2$ precursor structure attached to nascent glycoproteins, ER and Golgi α1,2-mannosidases catalyse the trimming of the four α1,2-linked mannose residues. The subsequent action of GlcNAc transferase I initiates complex chain formation and yields the substrate for Golgi α-mannosidase II which trims the terminal α1,3- and α1,6-mannose residues. In some tissues a distinct α-mannosidase trims $Man_5GlcNAc_2$ to $Man_3GlcNAc_2$ prior to the action of GlcNAc transferase I. Thereafter the N-glycan structure is further elaborated by Golgi glycosyltransferases.

α-Mannosidases have been classified into two groups based on amino acid sequence homology and on biochemical properties. Class I α-mannosidases specifically hydrolyze α1,2-linked mannose residues, and do not cleave substrates such as p-nitrophenyl-α-D-mannopyranoside. They require calcium for activity and are inhibited by 1-deoxymannojirimycin and kifunensine, but not by swainsonine. In contrast, Class II α-mannosidases can cleave α1,2-, α1,3- and α1,6-linked mannose residues as well as p-nitrophenyl-α-D-mannopyranoside and are inhibited by swainsonine, but not by 1-deoxymannojirimycin.

Although several mammalian α1,2-mannosidases that can remove up to four α1,2-mannose residues have been purified and cloned, there is significant biochemical evidence for the existence of highly

specific mammalian enzymes that trim $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ isomer B, the form lacking the middle-arm terminal α1,2-mannose, but mammalian enzymes with this specificity have not yet been purified or cloned. A mammalian ER α1,2-mannosidase that forms $Man_8GlcNAc_2$ isomer B and is not sensitive to 1-deoxymannojirimycin was described in intact UT-1 cells and in rat hepatocytes whereas distinct 1-deoxymannojirimycin-sensitive α1,2-mannosidase activity that processes $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ isomer B was observed in the ER of intact COS cells and in ER and in Golgi rat liver membrane preparations. Up to now the yeast ER processing α1,2-mannosidase is the only enzyme purified (Jelinek-Kelly and Herscovics, 1988) and cloned (Camirand *et al.*, 1991) that specifically trims $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ isomer B.

It would be highly desirable to be provided with the isolation, expression, and properties of a novel human cDNA encoding a Class I α1,2-mannosidase that specifically converts $Man_9GlcNAc$ to $Man_8GlcNAc$ isomer B.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide the isolation, expression, and properties of a novel human cDNA encoding a Class I α1,2-mannosidase that specifically converts $Man_9GlcNAc$ to $Man_8GlcNAc$ isomer B.

In accordance with the present invention there is provided a human α1,2-mannosidase enzyme for specifically converting $Man_9GlcNAc$ to $Man_8GlcNAc$ isomer B in degradation mechanism of misfolded proteins, wherein the enzyme has the characteristics of an enzyme encoded by a cDNA sequence set forth in Fig. 1.

In accordance with the present invention there is also provided the tools to develop specific agonist or antagonist of this particular α1,2-mannosidase that would not affect the other mannosidases.

The agonist or antagonist may provide for activating or inhibiting for a transient period of time. For example, such an antagonist may be

inhibiting the enzyme for a transient period of time, meanwhile preventing misfolded glycoproteins from being degraded.

In accordance with the present invention there is also provided the potential for a method for the treatment of genetic diseases causing misfolding of proteins in a patient, which comprises administering an antagonist of α1,2-mannosidase enzyme for transiently inhibiting the enzyme, thereby prevent misfolded glycoproteins from degradation.

For example, such a genetic disease includes, without limitation, cystic fibrosis, emphysema, among others.

The misfolded protein for cystic fibrosis is, for example, cystic fibrosis transmembrane conductance regulator (CFTR) (Ward et al., 1995).

The misfolded protein for emphysema is, for example, alpha1 antitrypsin.

For the purpose of the present invention the following abbreviations are defined below.

ER	endoplasmic reticulum;
RT	reverse transcriptase;
ORF	open reading frame;
RACE	rapid amplification of cDNA ends;
GSP	gene specific primer;
YPD	yeast peptone dextrose;
BMGY	buffered glycerol-complex;
BMMY	buffered methanol complex;
HPLC	high performance liquid chromatography.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the nucleotide and deduced amino acid sequence of the human α1,2-mannosidase cDNA;

Fig. 2 illustrates Northern blot analysis of human α1,2-mannosidase expression;

Fig. 3 illustrates the expression of the recombinant α1,2-mannosidase in *P. pastoris*;

Fig. 4 illustrates the time course human α1,2-mannosidase activity; and

Fig. 5 illustrates the [^{1}H]-NMR spectrum identifying the $Man_8GlcNAc$ isomer produced by the human α1,2-mannosidase.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided the isolation of a novel human cDNA encoding a type II membrane protein of 79.5 kDa with amino acid sequence similarity to Class I α1,2-mannosidases. The catalytic domain of the enzyme was expressed as a secreted protein in *Pichia pastoris*. The recombinant enzyme removes a single mannose residue from $Man_9GlcNAc$ and [^{1}H]-NMR analysis indicates that the only product is $Man_8GlcNAc$ isomer B, the form lacking the middle-arm terminal α1,2-mannose. Calcium is required for enzyme activity and both 1-deoxymannojirimycin and kifunensine inhibit the human α1,2-mannosidase. The properties and specificity of this human α1,2-mannosidase are identical to the endoplasmic reticulum α1,2-mannosidase from *Saccharomyces cerevisiae* and differ from those of previously cloned mammalian (mouse and human) Golgi α1,2-mannosidases that remove up to four mannose residues from $Man_9GlcNAc_2$ during *N*-glycan maturation. Northern blot analysis showed that all human tissues examined express variable amounts of a 3 kb transcript. This highly specific α1,2-mannosidase is likely to be involved in glycoprotein quality control since there is increasing evidence that trimming of $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ isomer B in yeast and mammalian cells is important to target misfolded glycoproteins for degradation. Furthermore, it has been shown that inhibition of this enzyme activity prevents the degradation of improperly folded mutant alpha1-antitrypsin characteristic of emphysema.

Materials

Oligonucleotides were synthesized by BioCorp (Montréal, Canada). $Man_9GlcNAc$ was prepared from soya bean agglutinin and [^{3}H]mannose-labeled $Man_9GlcNAc$ from rat liver as described previously (Jelinek-Kelly *et al.*, 1985; Bhattacharyya *et al.*, 1988). $Man_8GlcNAc$ isomer B substrate was prepared by digestion of $Man_9GlcNAc$ with the yeast α1,2-mannosidase. 1-deoxymannojirimycin, kifunensine and swainsonine were obtained from Toronto Research Chemicals, Inc. (Downsview, Canada). All other chemicals were reagent grade.

Isolation of a novel human α1,2-mannosidase cDNA

The NCBI dbEST database was searched with the yeast α1,2-mannosidase amino acid sequence (Camirand *et al.*, 1991) using the tBLASTn algorithm to identify novel α1,2-mannosidases. Retrieved ESTs were aligned using the DNASTAR SeqMan program (Madison, WI) and EST clones AA631254, R16652 and H46222 encoding the 3' region of the ORF were obtained from Genome Systems Inc. and sequenced. The following nested primers were designed based on the consensus sequence, 5'-CCACAGACCCAGCAAGGTGCC-3' and 5'-CTAGGCAGGGGTCCAGATAGG-3', and used to amplify clones containing additional 5' sequence from human placenta Marathon Ready cDNA (CLONTECH) according to the recommended protocol. A 5 µl aliquot the PCR reactions was subcloned into pCRII using the original TA cloning kit (Invitrogen) and clones encoding α1,2-mannosidase were identified by hybridization of colony lifts with gene specific ^{32}P-labeled oligonucleotide probes. The longest clones (2.1 kb) were sequenced.

Thereafter the Gibco 5' RACE System (Version 2) was used to isolate additional 5' sequence. First strand cDNA was synthesized from 2.5 µg of placenta, testis, and liver total RNA (CLONTECH) at 55°C using ThermoScript RT (Gibco) and a gene specific primer (GSP1 5'-GGGCACTTCTGCTCTTCTTGAAG-3') located within the 5' region of the

placenta cDNA clones. Nested 5' RACE amplicons were then generated using gene specific primers (GSP2 5'-ATGACTGTCCTCTGCGGATCTC-3', and GSP3.1 5'-TGTCTTCTGTGACGAAATCTC-3' or GSP3.2 5'-CAGAGCTTTCCAATGGTCAGC-3' or GSP3.3 5'-TCATAGCTCTCGCCA AAGCTCAGC-3') and Platinum *Taq* (Gibco) according to the recommended protocol. The amplicons were subcloned into pCR2.1 (Invitrogen), identified by hybridization of colony lifts with gene specific ^{32}P-labeled oligonucleotide probes, and sequenced. In addition, Genome Systems isolated three fetal brain α1,2-mannosidase cDNA clones (2.7 kb) by a PCR and hybridization cDNA library screen using primers located within the 5' ORF (5'-ATCGGGACTTCACCTCGGTG-3' and 5'-CAGAGC TTTCCAATGGTCAGC-3').

Northern blot analysis

The human α1,2-mannosidase EST R16652 was labeled with [α-^{32}P]dCTP (3000 Ci/mmol) using the multiprime DNA labeling kit (Amersham). The probe was hybridized to human multiple tissue Northern blots (CLONTECH) according to the recommended protocol and exposed to x-ray film for 5 days (Kodak).

Expression of the catalytic domain in Pichia pastoris

The DNA sequence encoding the soluble catalytic domain of the α1,2-mannosidase was amplified from a cDNA clone by PCR using the sense oligonucleotide 5'—AAAGAATTCCAGATTAGACCCCCAAGCCA AG-3' containing an EcoRI site and the antisense primer SHMSTOP 5'-AAATCTAGACTAGGCAGGGGTCCAGATAGG-3' containing the stop codon followed by an XbaI site. Expression vector pZαASHM169 was constructed by ligation of the amplicon into the EcoRI/XbaI sites of pPICZα A (Invitrogen) in frame with the α-factor secretion signal. Similarly, shorter untagged (pZαASHM240) and tagged (pZαASHM240T) expression constructs were prepared using sense primer 5'-AAAGA

ATTCCAGGGCACACCAGTGCATCTG-3' and antisense primers SHMSTOP, and SHMR 5'AAATCTAGAGCAGGGGTCCAGATAGGCAG-3' respectively. Primer SHMR lacks a termination codon thus the C-terminal of the recombinant enzyme was fused to the $(His)_6$ and Myc tags encoded by pPICZα A.

Pichia pastoris strain GS115 (*his4*) (Invitrogen) was transformed by electroporation with 10 μg of the expression constructs linearized with PmeI. Transformants were grown and assayed for α1,2-mannosidase activity as described previously.

α-Mannosidase Assays

To characterize the recombinant α1,2-mannosidase, medium containing the enzyme was concentrated 7.5 fold using centrifugal filters (Millipore) and equilibrated in 40 mM PIPES pH 6.5. Two microlitres of the concentrated medium was incubated with [^{3}H]mannose-labeled $Man_9GlcNAc$ at 37°C and the amount of released [^{3}H]mannose was determined by the Con A/PEG precipitation method.

Divalent cation requirements were studied by including 0.05 mM EDTA in duplicate assays in the absence or presence of 2 mM $CaCl_2$, $ZnCl_2$, $MnCl_2$, $MgCl_2$, and $CoCl_2$. The enzyme was incubated for 2 hours with 5000 cpm of [^{3}H]$Man_9GlcNAc$ in 40 mM PIPES pH 6.5, 1 mg/ml BSA, and 1 mM NaN_3.

The Km was determined by Lineweaver-Burk analysis. Duplicate 30 min assays contained $Man_9GlcNAc$ substrate (0.05-0.5 mM), 5000 cpm of [^{3}H]$Man_9GlcNAc$, 1 mM $CaCl_2$, 40 mM PIPES pH 6.5, 1 mg/ml BSA, and 1 mM NaN_3.

The effects of the Class I α-mannosidase inhibitors 1-deoxymannojirimycin and kifunensine, and the Class II α-mannosidase inhibitor swainsonine were investigated by preincubating the enzyme on ice for 30 min with the inhibitor in 40 mM PIPES pH 6.5, 1 mM $CaCl_2$, 1 mg/ml BSA , and 1 mM NaN_3. Substrate (0.8 mM $Man_9GlcNAc$ and 20,000

cpm of [^{3}H]$Man_9GlcNAc$) was then added to the duplicate assays and the mixtures were incubated at 37°C for 1 hour.

The time dependent formation of products was analyzed by assaying the recombinant enzyme at 37°C in a 30 µl mixture containing 3.4 mM $Man_9GlcNAc$, 30,000 cpm [^{3}H]$Man_9GlcNAc$, 44 mM potassium phosphate pH 6.5, 1 mg/ml BSA, 1 mM NaN_3 and 12 µl of unconcentrated medium. At 0, 1, 2, 4, 8, and 19.5 h one-sixth of the reaction mixture was collected. The products were resolved by HPLC, and identified by comparing their elution to that of the [^{14}C]$Glc_3Man_9GlcNAc$ internal standard, as described previously.

High Resolution [^{1}H]-NMR Analysis

Medium containing the human α1,2-mannosidase was incubated at 37°C with 600 µg $Man_9GlcNAc$ and 10^5 cpm [^{3}H]$Man_9GlcNAc$ in 44 mM potassium phosphate buffer pH 6.5 containing 1 mg/ml BSA and 1 mM NaN_3. The incubation was supplemented with additional enzyme after 8 and 22.5 h, and terminated after 28.5 h by boiling for 3 min. An aliquot of the sample was analyzed by HPLC to show that most of the sample was transformed to $Man_8GlcNAc$. The sample was then chromatographed on a Bio-Gel P-6 column (1 X 109 cm) equilibrated in deionized water. Fractions containing the oligosaccharide product were pooled, lyophilized, resuspended in D_2O and lyophilized four times, and stored over P_2O_5 in a vacuum desiccator. The [^{1}H]-NMR spectra were recorded at Université de Montréal NMR Facility in 5 mm tubes using a 600 MHz Bruker spectrometer at 30 and 70°C with the acetone chemical shift set to 2.225 ppm with respect to 4,4-dimethyl-4-silapentane sulfonate.

Polyacrylamide gel electrophoresis Western blotting

SDS-PAGE was performed using the Bio-Rad Mini-Protean II apparatus as described by Laemmli. Western blots were prepared by transferring proteins onto a nitrocellulose membrane (Schleicher and

Schuell), and expression of the Myc-tagged recombinant α1,2-mannosidase was detected using the monoclonal Anti-*myc* Antibody (Invitrogen) and visualized by the ECL Western blotting detection system (Amersham).

DNA Sequencing and Alignments

Manual sequencing was performed using the Pharmacia T7 and Deaza sequencing kits. The Sheldon Biotechnology Centre Automated Sequencing Facility (McGill University, Montréal, Canada) employed the ABI prism dye terminator and thermo sequenase fluorescent labeled primer cycle sequencing kits and the samples were run on the ABI 373A (Perkin Elmer) and ALFexpress (Amersham Pharmacia Biotech) sequencers respectively. The DNASTAR SeqMan program (Madison, WI) was used to assemble the sequences into contigs. The deduced amino acid sequences were aligned using the BestFit and Publish programs (Version 9.1) from the University of Wisconsin Genetics Computer Group (Madison, WI).

Isolation and characterization of a novel human α1,2-mannosidase *cDNA*

A novel human α1,2-mannosidase was identified by searching the EST database with the yeast α1,2-mannosidase amino acid sequence (Camirand *et al.*, 1991). The consensus sequence of the identified overlapping clones encodes the terminal 50 amino acids of the catalytic domain including two of the most highly conserved Class I α-mannosidase sequence motifs followed by 589 bp of 3' UTR terminating in a poly A tail. Clones encoding the complete ORF (2.1 kb) and 5' UTR were then isolated by nested 5' RACE from human placenta, liver and testis cDNAs, as described in materials and methods. In addition an independent 2.7 kb clone containing the ORF flanked by 50 bp of 5' UTR and 589 bp of 3' UTR terminating with a polyA tail was isolated from a human fetal brain

cDNA library. The sequence of the cDNAs obtained from the different sources were identical.

The 2.7 kb cDNA is predicted to encode a 79.5 kDa type II membrane protein with an 85 amino acid cytoplasmic tail, followed by a putative transmembrane domain of about 17 residues, a "stem" region of about 137 amino acids not required for enzyme activity and a large C-terminal catalytic domain (amino acids 240-699) (Fig. 1). The 5' UTR and ORF sequence were obtained from human placenta, testis and liver cDNA clones amplified by RACE, and a fetal brain cDNA library clone. The 3' UTR sequence is the consensus of an alignment of EST clones. Bold numbers refer to the deduced amino acid sequence and the numbers in normal type refer to the nucleotide sequence. The conserved Class 1 α-mannosidase sequence motifs are underlined, and the highly conserved invariant acidic amino acid residues as well as the conserved cysteines common to all class I α-mannosidases are boxed in grey. The putative transmembrane domain sequence is indicated in bold and underlined. Arrows indicate the starting amino acid residues of the different forms of the recombinant enzyme expressed in *Pichia pastoris*.

The cytoplasmic tail is much longer than any of the type II membrane-bound glycosidases or glycosyltransferases described so far, and contains a proline rich domain. The catalytic domain of this novel α1,2-mannosidase contains the highly conserved sequence motifs characteristic of Class I α-mannosidases as well as the highly conserved disulfide bonded cysteines and acidic amino acid residues that are essential for enzymatic activity. The catalytic domain of the human α1,2-mannosidase is 43% identical (54% similar) to the yeast α1,2-mannosidase (Camirand *et al.*, 1991), and 40% identical (54% similar) to human and murine α1,2-mannosidase IA and IB. There is no significant similarity between the N-terminal sequence of this novel α1,2-mannosidase and previously cloned members of the same family.

Northern blot analysis indicates that all tissues examined expressed variable levels of a 3 kb transcript (Fig. 2). Random labeled α1,2-mannosidase EST clone R16652 was hybridized to Northern blots containing 2 μg of poly (A^+) RNA from human tissue. The blots were exposed to film to x-ray film for 5 days.

The expression is particularly high in testis and relatively low in lung and muscle. The gene encoding this novel α1,2-mannosidase is localized on chromosome 9 since a clone (T12605) isolated from a human chromosome 9 cosmid library contains exonic sequence identical to the cDNA sequence (nucleotides 731-916).

Expression of recombinant α1,2-mannosidase in *Pichia pastoris*

The α1,2-mannosidase was expressed as a secreted protein in *P.pastoris* in order to characterize its enzymatic activity. The catalytic domain starting at either amino acid 169 or 240 was cloned in frame downstream from the α-factor of the *P.pastoris* expression vector pPICZα A. Following methanol induction of yeast transformed with expression constructs pZαASHM169, pZαASHM240 or pZαASHM240T similar levels of α1,2-mannosidase activity were detected in the medium, and absent in yeast transformed with the pPICZα A vector. Recombinant α1,2-mannosidase of the expected size (55 kDa) was observed by Western blot analysis at 2-3 days post-induction and decreased thereafter (Fig. 3). Ten microlitres of medium was subjected to reducing SDS-PAGE (10%) and the recombinant Myc tagged α1,2-mannosidase was visualized by Western blot analysis. Lanes 1-4 correspond to GS115 transformed with pZαASHM240T at 2, 3, 4, and 5 days post-induction, respectively. Lane 5 corresponds to GS115 transformed with vector at 5 days post-induction. Molecular mass standards are indicated on the right.

Properties of the human α1,2-mannosidas

The catalytic properties of the recombinant α1,2-mannosidase were studied using [^{3}H]$Man_9GlcNAc$ as substrate. The enzyme is active over a pH range of 6.3-7.2 with an optimum between 6.5-6.9. Inclusion of at least 0.1 mM Ca^{2+} in the assay is required to obtain maximum enzyme activity. The enzyme is inhibited 50% by 1 μM EDTA and 100% by 25 μM EDTA. This inhibition is completely reversed by the addition of 2 mM Ca^{2+} but, not by 2 mM Mn^{2+}, Mg^{2+}, Zn^{2+} or Co^{2+}. The K_m of the recombinant enzyme is 0.4 mM which is similar that of the recombinant yeast α1,2-mannosidase (0.3 mM).

The human α1,2-mannosidase is inhibited by the Class I α-mannosidase inhibitors, 1-deoxymannojirimycin (IC_{50} = 75 μM) and kifunensine (IC_{50} = 70 nM), but is insensitive to the Class II α-mannosidase inhibitor swainsonine (Table I).

Table I

Effect of α-mannosidase inhibitors on the human α1,2-mannosidase activity

Inhibitor	Activity[a] (% of control)
1-deoxymannojirimycin	
10 μM	84
50 μM	62
500 μM	10
kifunensine	
0.05 μM	67
0.10 μM	28
0.50 μM	0
swainsonine	
5 μM	100
10 μM	100
100 μM	100

[a] Assay conditions are described in materials and methods. Activity is expressed as a percentage of [^{3}H]mannose released in the absence of inhibitors (226 dpm).

Specificity of the human α1,2-mannosidas

The recombinant α1,2-mannosidase was incubated with [^{3}H]$Man_9GlcNAc$ for different periods of time and HPLC analysis showed that the only products formed are $Man_8GlcNAc$ and mannose in a time dependent manner (Fig. 4). [^{3}H]$Man_9GlcNAc$ was incubated with medium obtained from *P.pastoris* transformed with pZαASHM169 two days post-induction. Oligosaccharide product formation was monitored by HPLC as described in materials and methods and is expressed as a percentage of the total radioactivity recovered.

Supplementing the incubation mixture with fresh enzyme after 8 and 22.5 hours of incubation did not result in any further mannose trimming. The $Man_8GlcNAc$ oligosaccharide formed was demonstrated to be isomer B by [^{1}H]-NMR analysis (Fig. 5). Spectrum at 600 MHz and 30°C of the anomeric region of the $Man_8GlcNAc$ produced by the hydrolysis of $Man_9GlcNAc$ by the specific human α1,2-mannosidase. The resonance corresponding to the anomeric proton of each mannose residue is numbered. Integral values for each of the anomeric protons, except GlcNAc, were obtained. The split resonance signal at 5.104 and 5.075 ppm for residue 7 is characteristic of $Man_8GlcNAc$ isomer B.

Consistent with the strict specificity of this enzyme, $Man_8GlcNAc$ isomer B and p-nitrophenyl-α-D-mannopyranoside are not substrates of the enzyme. Therefore this highly specific α1,2-mannosidase is the human ortholog of the yeast processing α1,2-mannosidase that has been implicated in the targeting of misfolded glycoproteins for degradation (Knop *et al.*, 1996; Jakob *et al.*, 1998). Since there is evidence that *N*-glycan trimming by 1-deoxymannojirimycin and kifunensine-sensitive ER α1,2-mannosidase activity is also implicated in the degradation of misfolded glycoproteins in mammalian cells (Su *et al.*, 1993; Liu *et al.*, 1997; Yang *et al.*, 1998; Liu *et al.*, 1999), it seems likely that the novel

human α1,2-mannosidase we have cloned is involved in ER quality control, but this remains to be shown.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Possible approaches whereby cloning and expression of thisnew human α1,2-mannosidase can be used to design new specific inhibitors that might be developed for drug therapy of glycoprotein folding diseases:

(1) use of recombinant enzyme for high throughput screening of naturally occurring compounds as potential specific inhibitors.

(2) use of recombinant enzyme to test chemically synthesized derivatives of mannodeoxynojirimycin and kifunensine as potential specific inhibitors.

(3) molecular modelling of the human mannosidase based on its amino acid sequence and the recently determined three-dimensional structure and identification of the active site of the yeast α1,2-mannosidase ortholog that has the same properties, specificity and function in degradation of misfolded glycoproteins.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A human α1,2-mannosidase enzyme for specifically converting $Man_9GlcNAc$ to $Man_8GlcNAc$ isomer B in degradation mechanism of misfolded proteins, wherein said enzyme has the characteristics of an enzyme encoded by a cDNA sequence set forth in Fig. 1.

2. An agonist or antagonist of the α1,2-mannosidase enzyme of claim 1 for activating or inhibiting said enzyme.

3. The agonist or antagonist of claim 2, wherein said activating or inhibiting is for a transient period of time.

4. An antagonist of claim 2, wherein said inhibiting is for a transient period of time, thereby preventing degradation of misfolded glycoproteins.

5. A method for the treatment of a genetic disease causing a misfolding of proteins in a patient, which comprises administering an antagonist of α1,2-mannosidase enzyme of claim 1 for transiently inhibiting said enzyme, thereby preventing degradation of misfolded glycoproteins.

6. The method of claim 5, wherein said genetic disease is selected from the group consisting of cystic fibrosis and emphysema.

7. The method of claim 6, wherein for cystic fibrosis said misfolded protein is cystic fibrosis transmembrane conductance regulator (CFTR).

8. The method of claim 6, wherein for emphysema said misfolded protein is alpha1 antitrypsin.

ABSTRACT OF THE INVENTION

The present invention also relates to the use of the recombinant α1,2-mannosidase for the development of specific agonist or antagonist of the specific α1,2-mannosidase enzyme for specifically converting $Man_9GlcNAc$ to $Man_8GlcNAc$ isomer B in degradation mechanism of misfolded proteins, wherein the enzyme has the characteristics of an enzyme encoded by a cDNA sequence set forth in Fig. 1. The present invention also relates to an agonist or antagonist of the α1,2-mannosidase enzyme of claim 1 for activating or inhibiting the enzyme. The present invention also relates to a method for the treatment of genetic diseases resulting in misfolding of glycoproteins in a patient, which comprises administering an antagonist of α1,2-mannosidase enzyme of claim 1 for transiently inhibiting the enzyme, thereby stabilizing misfolded glycoproteins and preventing their degradation.

Applicant or Patentee: Annette A. HERSCOVICS et al.
Serial or Patent No.: _____ Atty. Dkt. No.: 1770-228"USPR" FC/ld
Filed or Issued: _____
For: α1,2-MANNOSIDASE AND THERAPEUTICAL USES THEREOF

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS [(37 CFR 1.9(f) AND 1.27 (d)] - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION McGILL UNIVERSITY
ADDRESS OF ORGANIZATION 845 Sherbrooke Street West, Montréal, Québec Canada H3A 2T5

TYPE OF ORGANIZATION

(X) UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
( ) TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE [26 USC 501(a) AND 501(c)(3)]
( ) NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)
( ) WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE [26 USC 501(a) AND 501 (c)(3)] IF LOCATED IN THE UNITED STATES OF AMERICA
( ) WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE _____)
(CITATION OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9 (e) for purposes of paying reduced fees under section 41(a) or (b) of Title 35, United States Code with regard to the invention entitled α1,2-MANNOSIDASE AND THERAPEUTICAL USES THEREOF by inventor(s) Annette A. HERSCOVICS et al. described in

(X) the specification filed herewith
( ) application serial no. _____, filed _____
( ) patent no. _____, issued _____.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. [37 CFR 1.27]

NAME _____
ADDRESS _____
( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN ( ) NONPROFIT ORGANIZATION

NAME _____
ADDRESS _____
( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN ( ) NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28(b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Alex Navarre
TITLE IN ORGANIZATION Director – Office of Technology Transfer
ADDRESS OF PERSON SIGNING 845 Sherbrooke Street West, Montréal, Québec, Canada H3A 2T5

SIGNATURE _____ DATE _____

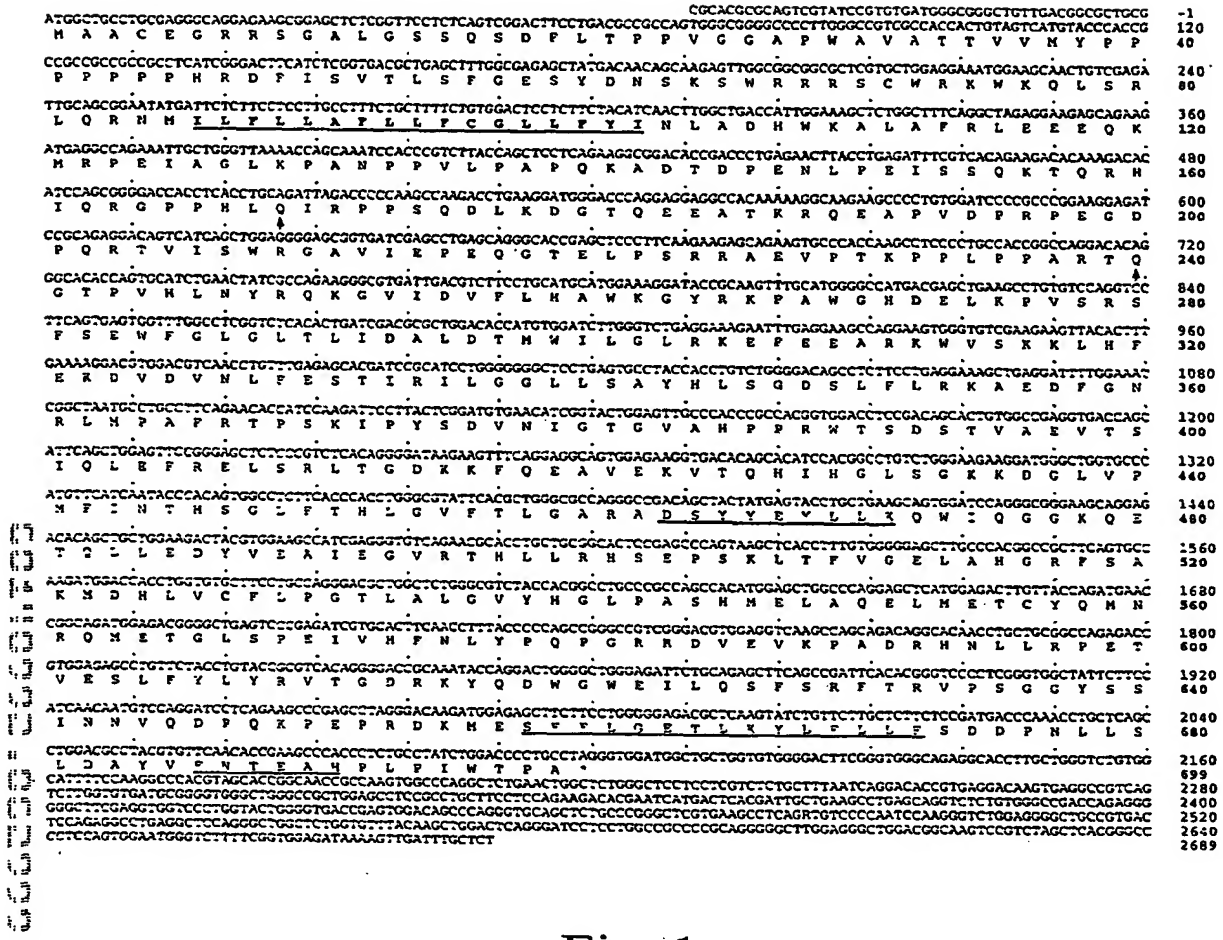

Fig. 1

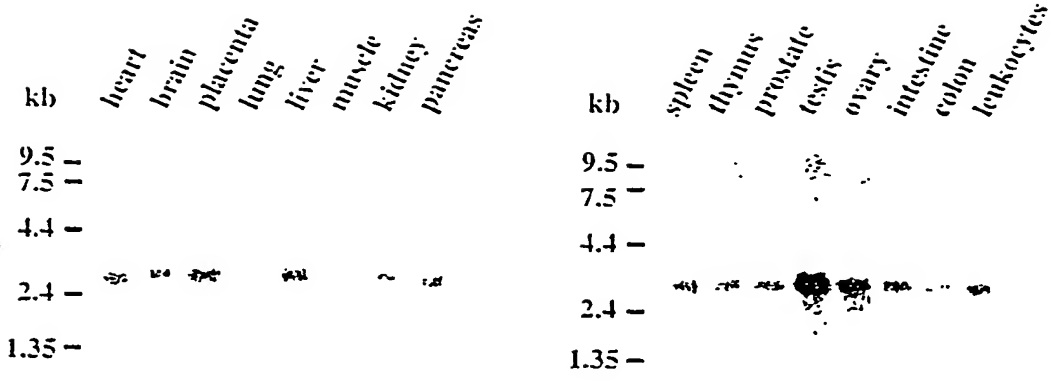
kb
heart
brain
placenta
lung
liver
muscle
kidney
pancreas
9.5
7.5
4.4
2.4
1.35
kb
spleen
thymus
prostate
testis
ovary
intestine
colon
leukocytes
9.5
7.5
4.4
2.4
1.35

Fig. 2

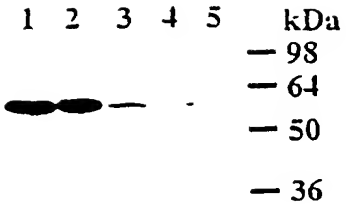

Fig. 3

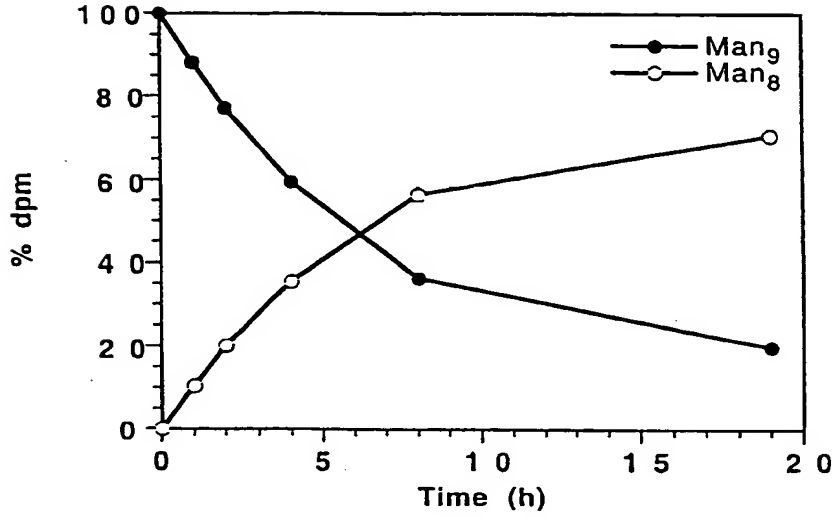

Fig. 4

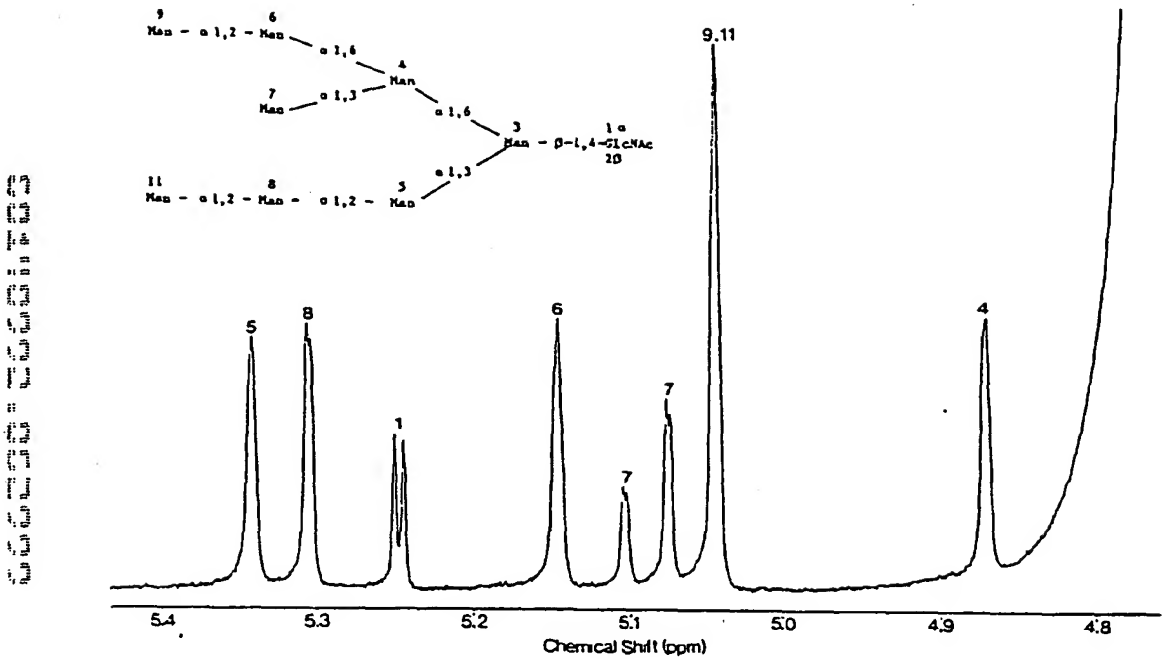
9
Man - α 1,2 - 6
Man
α 1,6
4
Man
7
Man
α 1,3
α 1,6
3
Man - β-1,4-GlcNAc
1α
2β
α 1,3
11
Man - α 1,2 - 8
Man - α 1,2 - 5
Man
9,11
5
8
1
6
7
7
4
5.4
5.3
5.2
5.1
5.0
4.9
4.8
Chemical Shift (ppm)

Fig. 5